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Purification and characterisation of two forms of toxin B produced by *Clostridium difficile*

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Toxin B from *Clostridium difficile* was purified to homogeneity by gel filtration and high resolution ion exchange chromatography. Two forms of toxin B were found. Form 1 which seemed to consist of two identical subunits of 220–300 kDa; femtogram amounts of this toxin induced rounding of fibroblast cells. Form 2 contained subunits of 43 kDa and 105 kDa; the stoichiometric ratio probably being 4 : 1; picogram amounts were needed to induce rounding of fibroblast cells. Immunological studies suggested that both subunit types were antigenic and had epitopes which were identical with those of form 1.

Cytotoxin; Diarrhea; Colitis; (*Clostridium difficile*)

1. INTRODUCTION

Clostridium difficile has been reported in association with cases of diarrhoea, colitis and pseudomembranous colitis in humans [1,2]. The bacterium produces two toxins, A and B, and these proteins probably mediate most of the pathogenic effects [3,4]. Toxin A is an enterotoxin causing intestinal fluid secretion in animal models; toxin B is a cytotoxin causing cell damage in tissue cultures. Both toxins are lethal to mice after intraperitoneal injection of <1 μg.

Toxin B has proved to be difficult to purify to homogeneity, a property which has hindered its characterisation. Most reports agree that it is a large protein with a native M_r from 400 000 to 600 000 and subunits with an M_r between 200 000 and 300 000 [3,4]. However, a toxin with subunits of smaller size (50 kDa) has also been reported [5,6], but the purity of these preparations has been questioned [7]. In this report, we provide chemical and immunological evidence that both forms of toxin B exist and are produced by the same bacterial strain.

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2. MATERIALS AND METHODS

2.1. *Culture conditions*

C. difficile CCUG 19126 (VPI 10463, obtained from DM Lyerly/TD Wilkins, VPI Blacksburg, USA) was used for toxin production. The organism was grown in 2-l brain heart infusion dialysis flasks, as described [8]. Culture was incubated at 37°C for 96 h, and a cell-free supernatant was obtained by centrifugation and filtration.

2.2. *Purification of toxin B*

An overview of the various procedures used for the purification of toxin B is shown in table 1. The culture filtrate was concentrated in an ultrafiltration cell with an XM100A membrane (Amicon, USA), and toxin B was separated from toxin A in DEAE-Sephadex A 25 as outlined [3]. Fractions with toxin B activity were concentrated to 5–6 ml, by ultrafiltration using an XM50 membrane (Amicon), and applied onto a 2.6 × 76 cm column of Sephacryl S400 (Pharmacia, Sweden). Elution was done with 0.05 M triethanolamine, pH 7.5, containing 0.2 M NaCl. Fractions with cytotoxic activity were concentrated and equilibrated in triethanolamine 0.2 M, pH 7.5 (buffer 1), by washing 3 times with the buffer on an ultrafiltration XM50 membrane. The sample was then applied onto a 5 mm × 5 cm Mono QHR 5/5 column (Pharmacia), coupled to a fast protein

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Table 1

Purification of form 1 of toxin B produced by *C. difficile*

Purification step	Protein			Cytotoxicity[a]	
	vol., ml	mg/ml	Total mg	Total CU	CU/pg
Cell-free culture	300	3.93	1180	9.5×10^{12}	8
DEAE-Sephadex A25	10	7.90	55	6.7×10^{12}	120
Sephacryl S-400	25	0.37	9.2	5.8×10^{12}	620
Mono Q, FPLC[b]	5.0	0.22	1.1	4.6×10^{12}	4200

[a] Expressed as cytotoxic units (CU) i.e. the inverse of the maximal dilution which induced rounding of human lung fibroblast cells
[b] Fast protein liquid chromatography on a Mono Q column as shown in fig.1

liquid chromatography (FPLC) system (Pharmacia), the flow rate being 0.75 ml/min and the fraction volume 1 ml. After an initial washing with 0.44 M NaCl in buffer 1, the toxin was eluted in a gradient of 0.44 to 0.52 NaCl in buffer 1 (fig.1). Samples with toxin B activity were concentrated to about 1 mg/ml of protein, and kept at 4°C for further analysis.

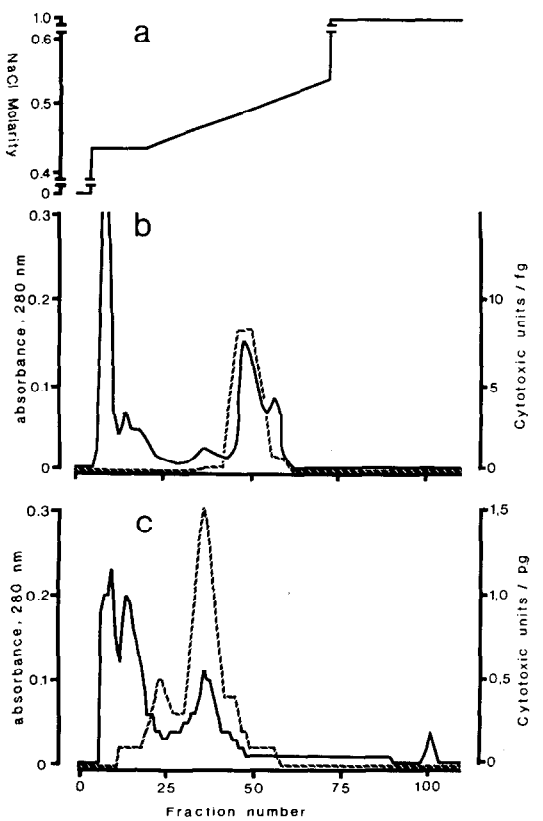

Fig.1. Fast protein liquid chromatography of toxin B on a Mono Q column. (a) NaCl gradient used for elution. (b) Elution of toxin B form 1. (c) Elution of toxin B, form 2. ——, absorbance at 280 nm; – – – –, cytotoxic activity.

2.3. *Toxicity assay*

Human lung fibroblast cells were used to measure the activity of toxin B, as described [9]. Cytotoxic units are expressed as the inverse of the maximal dilution which induced rounding in at least 50% of the cells.

2.4. *Polyacrylamide gel electrophoresis (PAGE)*

Samples were analysed in precast polyacrylamide gradient gels (Pharmacia). Non-denaturing PAGE was run in a 4–30% gradient at pH 8.4, in accordance with the manufacturer's instructions. Some unstained gels were sliced into sections, and the active toxin eluted by dialysis against phosphate buffered saline, containing 0.15 M NaCl, 0.1% sodium thiosulphate, and 0.01 M sodium phosphate, pH 7.2. Denaturing PAGE in the presence of SDS was run at pH 7.4, and a 2–16% gradient gel (as recommended by Pharmacia). The gels were stained with 0.1% Coomassie blue R-250. Some gels were scanned in a dual-wavelength densitometer (Shimadzu CS-910) to determine the proportion of each stained spot.

2.5. *Immunological methods*

New Zealand rabbits were used to prepare antisera against toxin B. Animals were initially inoculated with 20 μg of protein in complete Freund's adjuvant by multiple intradermal dorsal injection. 10 days later, they were inoculated with 20 μg of protein in incomplete Freund's adjuvant, again by multiple intradermal dorsal injection. This procedure was repeated 8 times. Eight days after the last injection, animals were bled to death and the serum prepared.

The antigen-antibody reaction was analysed by double diffusion-in-gel [10]. 10–20 μg of antigen was used in each well, and the antiserum was undiluted. After 48 h of diffusion, the microplate was washed with 0.15 M NaCl and stained with Coomassie blue R-250.

3. RESULTS

The crude culture filtrate contained between 5 and 10×10^{12} cytotoxic units per ml (CU/ml) of toxin B. The toxin was purified to homogeneity by chromatography successively on DEAE-Sephadex, Sephacryl S-400 and Mono Q (table 1). The DEAE-Sephadex step separated toxin B from toxin A, giving a 15-fold increase in specific activity of

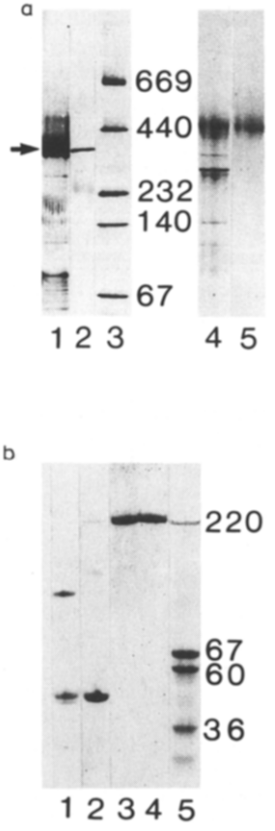

Fig.2. Polyacrylamide gel electrophoresis of toxin B. (a) Electrophoresis under non-denaturing conditions; rows 1 and 2, toxin B form 2, before and after FPLC, respectively; row 3, molecular mass markers; rows 4 and 5, toxin B form 1 before and after FPLC, respectively. Arrows on both sides indicate the zone from which activity was recovered. (b) Electrophoresis in the presence of SDS; rows 1 and 2, toxin B form 2 without and with β-mercaptoethanol, respectively; rows 3 and 4, toxin B form 1 without and with β-mercaptoethanol, respectively; row 5, molecular mass markers.

toxin B. The subsequent filtration through Sephacryl S-400 increased the specific activity about five times with a yield of 80–90%.

The final purification step was high performance liquid chromatography (FPLC) on Mono Q, using a combination of discontinuous and continuous NaCl gradients (fig.1). Most of the contaminating proteins eluted before toxin B which eluted at 0.48–0.49 M NaCl. The specific activity increased about 7 times in this step, and the yield was about 80%. In all, the 3 steps gave 500–600-fold purification, and a yield of about 50%. Some lots of toxin B (form 2) behaved differently on FPLC, the toxin eluting at 0.46–0.47 M NaCl (fig.1c). The yields and specific activity before FPLC were similar for the two forms, but after FPLC form 2 had less activity than form 1. Both forms of toxin were analysed with gradient PAGE (fig.2). Under non-denaturing conditions, form 1 appeared at a position representing 450–500 kDa (row 5), but form 2 at 300–400 kDa (row 2). Further, the toxic activity was entirely recovered from these regions (fig.2a, arrows), when the unstained gel was sliced into small pieces and eluted with buffer. In SDS-PAGE, the two toxins differed substantially in their subunit composition (fig.2b). Form 1 migrated in SDS as one band of 220–300 kDa, both in non-reduced (row 3) and reduced conditions (row 4). In contrast, the non-reduced form 2 migrated in two bands of molecular masses of 105 and 45 kDa, containing 34 and 63% of stained material, respectively (row 1). In reducing conditions, form 2 migrated as one component of 43 kDa (row 2). These results suggest that form 1 is composed of two subunits of about 250 kDa; form 2 is probably composed of four smaller subunits and one larger subunit, the larger being an S-S bridged dimer of two peptides, similar in mass to the smaller subunit. In one

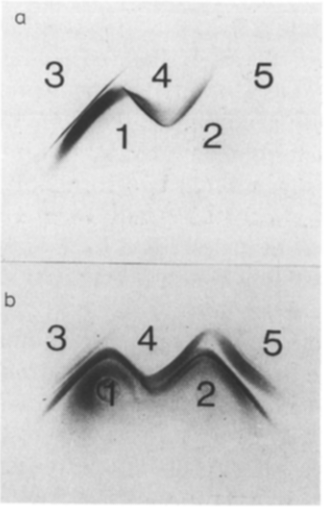

Fig.3. Analysis in double immunodiffusion-in-gel of toxin B. (a) Pos.1, antiserum against toxin B form 1; pos.2, antiserum against toxin B form 2; pos.3 and 4, toxin B form 1, before and after FPLC, respectively; pos.5, toxin A. (b) Pos.1, antiserum against toxin B form 1; pos.2, antiserum against toxin B form 2; pos.3, toxin B form 1; pos.4 and 5, toxin B form 2.

preparation of the toxin, two bands were obtained in gradient PAGE at positions corresponding to form 1 and form 2 (not shown); SDS-PAGE in the presence of mercaptoethanol revealed the existence of subunits of molecular masses of 230 and 43 kDa (i.e. the subunits of form 1 and form 2).

Two different antisera against toxin B were prepared, one against form 1 and the other against form 2. The former antiserum had a high neutralisation capacity; 1 ml neutralised 10^7 CU of toxin B form 1, whereas the antiserum against form 2 neutralised only 10^2 CU/ml. Neither of the antisera showed any inhibition of toxin A. The antigen-antibody reaction was further analysed by immunodiffusion. As shown in fig.3a, the pure form 1 precipitated in one line both with antisera against form 1 and against form 2; the antiserum against toxin B did not recognise toxin A. As shown in fig.3b, toxin B, form 2, precipitated in two lines with its own antiserum, both lines fusing with that of form 1. This suggests that form 2 is composed of two antigenically different types of subunits, both of which have antigenic determinants in common with form 1.

4. DISCUSSION

Using the 10463 strain of *C. difficile* Lyerly et al. [11] and Pothoulakis et al. [6] obtained preparations of toxin B which differed in their chemical properties. Thus, the former group reported that the toxin consisted of subunits of M_r 300 000 while the latter determined the subunit size at 50 000. Our results suggest that two forms of toxin B are produced by the 10463 strain: form 1 is probably identical with that described by Lyerly et al. and form 2 is probably identical with that described by Pothoulakis et al.

Comparison of the two forms in immunodiffusion revealed that the antigenic determinants of the two forms were similar, if not identical. Form 2, however, in contrast to form 1, contained two precipitogens which probably corresponded to the 105 kDa and 50 kDa subunits seen in SDS-PAGE. Interestingly, a similar toxin was recently isolated from *C. sordelli* broth [12]. This toxin cross-reacted antigenically with toxin B and was composed of about five subunits each of a molecular mass of 50 kDa.

The existence of different forms of one toxin has also been reported in a species of *Bacillus*. In *B. sphaericus*, for instance, toxic peptides varying from 110 to 39 kDa have been purified [13], and proteases have been suggested to hydrolyse the intact toxin into smaller polypeptides. Again, in *B. thuringiensis* two similar 130 kDa mosquitocidal toxins are probably encoded by two independent genes [14]. Further studies are needed to clarify whether the forms of toxin B reported here are encoded by different genes, or the result of the action of hydrolysing enzymes.

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